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Original Article

**Protein Phosphatase 2C of *Toxoplasma Gondii* Interacts with Human SSRP1 and Negatively Regulates Cell Apoptosis***

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Abstract

Objective The protozoan *Toxoplasma gondii* expresses large amounts of a 37 kDa Type 2C serine-threonine phosphatase, the so-called TgPP2C which has been suggested to contribute to parasite growth regulation. Ectopic expression in mammalian cells also indicated that the enzyme could regulate growth and survival. In this study, we aimed to investigate the interaction of TgPP2C with human SSRP1 (structure-specific recognition protein 1) and the effects of TgPP2C on cell viability.

Methods The yeast two hybrid system, His-tag pull-down and co-immunoprecipitation assays were used to confirm the interaction of TgPP2C with SSRP1 and determine the binding domain on SSRP1. The evaluation of cell apoptosis was performed using cleaved caspase-3 antibody and Annexin-V/PI kit combined with flow cytometry.

Results We identified human SSRP1 as an interacting partner of TgPP2C. The C-terminal region of SSRP1 including the amino acids 471 to 538 was specifically mapped as the region responsible for interaction with TgPP2C. The overexpression of TgPP2C down-regulated cell apoptosis and negatively regulated apoptosis induced by DRB, casein kinase II (CKII) inhibitor, through enhanced interaction with SSRP1.

Conclusion TgPP2C may be a parasitic factor capable of promoting cell survival through interaction with the host protein SSRP1, thereby creating a favorable environment for parasite growth.

Key words: Apoptosis; SSRP1; TgPP2C; *Toxoplasma gondii*

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INTRODUCTION

In eukaryotes, PP2C is one of the four major conserved serine/threonine phosphatases (PP1, PP2A, PP2B, and PP2C)^[1-2]. At least 15 different gene products of PP2C have been identified in human that encode the superfamily of PP2Cs, which seems to arise following a series of duplication events^[3]. PP2C family members play critical roles in cell survival and apoptosis regulation^[4]. The majority of them are involved in regulation of stress activated protein kinase (SAPK) cascades^[5] at different levels of the pathway. For example, PP2C α , PP2C β , PP2C ϵ , and Wip1 have been shown to negatively regulate cell apoptosis by inhibiting SAPK signaling pathways^[6-9]. In contrast, PP2C α and PP2C β promoted cell apoptosis of neuronal and endothelial cells^[10], whereas PP2C δ /ILKAP promoted cancer cell apoptosis by stimulating TNF α -induced signaling pathways^[4]. On the other hand, the PP2C superfamily is also associated with eukaryotic cell cycle processes in particular by controlling the CDKs phosphorylation status^[11-12]. Wip1 can dephosphorylate p53 and negatively regulates the p36-p53 pathway mediating the cell cycle arrest and apoptosis induced by UV irradiation^[13]. In yeast, it has been reported that the overexpression of Ptc2 and Ptc3 may, by the dephosphorylating Rad53, relieve cell division arrest caused by DNA damage^[14]. These results suggested a positive role of PP2C family on cell survival.

Toxoplasma gondii (*T. gondii*) is a protozoan eukaryote member of the Apicomplexa phylum. It is an obligate intracellular parasite which can cause anthroponosis of various severities and can be even lethal in immuno-suppressed hosts. The tachyzoite stage of *T. gondii* invades and develops in any nucleated cell. Previous studies showed that *T. gondii* infection protected its host cells from apoptosis induced by multiple apoptotic stimuli, including UV or gamma irradiation, growth factor scarcity, IL-2 deprivation, and exposure to toxins^[15-16]. The precise mechanism by which the parasite establishes such anti-apoptotic condition in the host cells remains unclear. However, it has been suggested that a phosphatase of *T. gondii* might be involved in the establishment of the anti-apoptotic condition in a variety of cell types^[17]. In extracellular and intracellular tachyzoites, previous studies have pinpointed that type 2C protein phosphatases represent the major type of serine threonine phosphatase activities: two molecular species have been shown to be particularly abundant and called

hnPP2C^[18] and TgPP2C^[19]. While hnPP2C is secreted into the cytoplasm of the host cells and translocate to the host nucleus during infection^[18], there are data supporting TgPP2C being secreted as well (our unpublished data). In addition, ectopic expression in mammalian cells shows that TgPP2C interferes with the cell activity by promoting G2/M arrest. Collectively, these data prompted us to investigate the physiological role of TgPP2C in the host cell, particularly its possible involvement in the regulation of cell apoptosis.

In order to assess the physiological functions of TgPP2C in host cells, we have previously used yeast two hybrid system to identify the cellular proteins interacting with it (our unpublished data). One of the candidate proteins is SSRP1 (structure-specific recognition protein), initially identified as a cisplatin-modified DNA binding protein and played an important role in the regulation of gene replication and transcription^[20]. SSRP1 was required for cell viability and early embryonic development^[21]. This feature incited us to investigate if the potential interaction between these two proteins could be important for the determination of cell fate.

In this study, we reported SSRP1 as an interacting partner of TgPP2C. The interaction has been initially identified in Yeast two-hybrid system and then confirmed by the biochemical approaches. We also determined that the C terminal region of SSRP1 as sufficient to promote the interaction with the phosphatase, and provided evidence that TgPP2C-SSRP1 interaction is important for the control of cell fate.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa cells (Cell Resource Center, Institute of Life Science Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand island, NY) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria), 1% penicillin/streptomycin (Genom, China) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells at 80%-90% confluence in a 6-well plate were transfected with 2 μ g of DNA using Lipofectamin 2000 according to the manufacturer (Invitrogen, Carlsbad, CA).

Yeast Strain

The yeast strain L40 (MATa *leu2 his3 trp1 ade2*

GAL4 gal80, *LYS2::(lexAop)4-HIS3 URA3::(lexAop)s-lacZ*) was provided by Dr. Serge Benichou. The yeast was grown at 30 °C in either YPAD (Yeast Extract-Peptone-Adenine-Dextrose) medium or SC-Leu-Trp (synthetic complete yeast growth medium lacking leucine and tryptophan) medium to maintain plasmids.

Construction of Hybrid Plasmids for Yeast Two-hybrid Study

The full length cDNA of TgPP2C were amplified by PCR from HA-TgPP2C (provided by Dr. Isabelle Tardieux at Université Descartes of France) using the primers (forward: 5'-CAGGAATTCGGATGAAGTCCTC TGCTGAAATT-3', reverse: 5'-GTGCTCGAGGCTAATC AGTCTTCTTGAAG-3') and cloned in-frame into *XhoI-EcoRI* sites in the yeast expression vector pLEX12. The entire or truncated versions of SSRP1 were amplified by PCR from pCDNA3.1-SSRP1 (provided by Prof. Hua Lu at Indiana University of Indiana) using the following appropriate primers flanked by appropriate restriction sites and ligated in-frame into the yeast expression vector pGAD-GE. The primers for pGAD-GE-SSRP1 are 5'-TTACTCG AGTATGGCAGAGACACTGGAGTTC-3' and 5'-TGTTCTA GAACTACTCATCGGATCCTGACGC-3'; and the restriction enzymes for it are *XbaI* and *XhoI*. The primers for pGAD-GE-N-SSRP1 are 5'-GTCGGATCCATATGGCA GAGACACTGGAGTTC-3' and 5'-GAACTCGAGGACTAC AGACGCAGTACTGTGGT-3'; and the restriction enzymes for it are *BamHI* and *XhoI*. The primers for pGAD-GE-M-SSRP1 are 5'-CTCGAATCCCCTACACCAC AGTACTGCGT-3' and 5'-GCTTCTAGACGCTATCCTGAG TCATCGTGCT-3'; and the restriction enzymes for it are *EcoRI* and *XbaI*. The primers for pGAD-GE-C-SSRP1 are 5'-CAGGAATTCAGCGATGACTCAGGA GAAGAAG-3' and 5'-TTACTCGAGGTCTACTCATCGGA TCCTGAC-3'; and the restriction enzymes for it are *EcoRI* and *XhoI*. The primers for pGAD-GE-C1-SSRP1 are 5'-CAGGAATTCAGCGATGACTCAGGAGAAGAAG-3' and 5'-TTACTCGAGAGCTACACCTCCACAGGCTTCTT-3'; and the restriction enzymes for it are *EcoRI* and *XhoI*. The primers for pGAD-GE-C2-SSRP1 are 5'-CAGGAA TTCAAGAAGGGCAAAGACCCCAAT-3' and 5'-GGACTC GAGAGCTAATATCTTTCATGGCT-3'; and the restriction enzymes for it are *EcoRI* and *XhoI*. The primers for pGAD-GE-C3-SSRP1 are 5'-TATGAATTCGAAGGG GGCCGAGGCGAGTCT-3' and 5'-TTACTCGAGGTCTACT CATCGGATCCTGAC-3'; and the restriction enzymes for it are *EcoRI* and *XhoI*. The yielded hybrid plasmids were then verified by DNA sequencing. The yeast expression vector pLEX12 carried the BD fusion and

the *TRP1* marker. The yeast expression vector pGAD-GE carried the AD fusion and the *LEU2* marker.

Yeast Two-hybrid Analysis of Protein-protein Interaction

Two hybrid plasmids were introduced into the yeast strain *L40* by the lithium acetate transformation. Transformants were grown on SC-Leu-Trp (synthetic complete yeast growth medium lacking leucine and tryptophan) selective medium at 30 °C for 2 to 3 d. The produced colonies were picked and patched onto a single SC-Leu-Trp plate. After incubation for 18 h at 30 °C, the colonies on this plate were transferred to the YPAD plate containing a nylon membrane (Shanghai Bandao Industrial Co., Ltd., China) and incubated for 24 h at 30 °C for the X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) assay.

X-gal Assay

X-gal assay was used to examine the expression of the lacZ reporter gene in *L40* yeast reporter strain. Briefly, the nylon membrane with the patches of yeast was carefully removed from the surface of the YPAD plate, frozen in liquid nitrogen for 30 s, and then placed on a plate containing X-gal solution (for each membrane, 10 mg X-gal was dissolved in 100 μ L DMF, 60 μ L 2-mercaptoethanol and 10 mL Z buffer). The covered plate was then incubated at 37 °C to check for the appearance of blue color and monitor the β -Galactosidase enzyme activity over a 24 h period.

Construction, Expression and Purification of His-TgPP2C Fusion Protein

Construction of pBAD-His-TgPP2C expression vector: the full length TgPP2C was amplified by PCR from HA-TgPP2C plasmid and inserted into pBAD-His A vector (kindly provided by Prof. Xuesong Sun of Jinan University, China) with *XhoI* and *HindIII* to construct the pBAD-His-TgPP2C recombinant expression vector. The PCR primers were 5'-GTCCTC GAGAAGTCCTCTGCTGAAATT-3' and 5'-GCGAAGCT TGCCTAATCAGTCTTCTTGAAC-3'. The presence of the insert in frame was verified by DNA sequencing. His-TgPP2C fusion protein was expressed in *E. coli* BL21 after induction with 20% L-arabinose for 8 h at 25 °C. To subsequently purify pBAD-His- TgPP2C, bacteria were resuspended in lysis buffer A [50 mmol/L Tris-HCL, pH7.6, 150 mmol/L NaCl,

1% Triton X-100, 1% protease inhibitor cocktail (Roche), 2 mmol/L PMSF] containing 100 µg/mL lysozyme. Bacterial extracts were sonicated for 20 min and centrifuged at 12,000 rpm for 30 min at 4 °C to remove cell debris. His-TgPP2C fusion protein was purified from supernatant of bacterial lysates by affinity chromatography using Ni-NTA Superflow according to the manufacturer (Qiagen). The concentration of purified protein was quantified by a BCA assay (Beyotime Biotechnology, China).

His-tag Pull-down Assay

Pull-down assay was performed to assess the interaction of His-tagged TgPP2C protein with the target proteins in HeLa cells. To prepare cell lysate for His-tag pull-down assay, untreated HeLa cells or 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB, Sigma, St. Louis, MO) -treated cells (100 µmol/L, 6 h) were harvested and resuspended in ice-cold lysis buffer A for 30 min on ice. Then, the cell suspension was centrifuged at 13,200 rpm for 30 min at 4 °C. The protein concentration was determined by BCA protein assay kit.

The cell extract was precleared by incubation with 20 µL of 50% Ni-NTA Superflow for 20 min twice at 4 °C on a rocker. The precleared supernatants (500 µg of proteins) were then incubated with Ni-NTA Superflow beads coated or not with His-TgPP2C (30 µg) respectively for 4 h at 4 °C on a rocker. After brief centrifugation, the beads were washed four times with lysis buffer A, resuspended and boiled in SDS-PAGE sample buffer. Then, the fractions bound to Ni-NTA Superflow or His-TgPP2C were subjected to SDS-PAGE and analyzed by standard immunoblotting using specific mouse monoclonal antibody against SSRP1 (Clone10D1, BioLegend). The levels of recombinant His-TgPP2C proteins used were analyzed by His (AB102-01, Tiangen, China) antibody or CBB (Coomassie Brilliant Blue) staining of SDS-PAGE gels.

Co-immunoprecipitation Assay

Pellets of cell were lysed in buffer B (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na₃VO₄, leupeptin, and 1% protease inhibitor cocktail (Roche). The extracts were precleared with 20 µL protein A/G-agarose (Santa Cruz) and 1 µg mouse IgG on a rocker at 4 °C for 30 min. After that, the precleared supernatant were incubated with the mouse monoclonal HA

antibody (H9658, Sigma) at 4 °C overnight with gentle agitation. The immune complexes were then analyzed by immunoblotting using specific antibodies against HA and SSRP1 as described below.

Western Blot Analysis

The level of cleaved caspase-3 were analyzed by Western blotting in HeLa cells expressing HA-TgPP2C and/or treated with DRB (100 µmol/L, 6 h). Whole cell extracts were prepared with the lysis buffer B. The same amounts of protein samples (as determined by a BCA assay) were separated by 10% SDS/PAGE, electrophoresed and transferred onto a PVDF membrane. Blots were probed with the Cleaved Caspase-3 antibody (Cell Signaling Technology), mouse monoclonal HA antibody (H9658, Sigma), and mouse monoclonal GAPDH antibody (ZS-25778, ZSGB-BIO, China) at 4 °C overnight with gentle agitation. Standard immunoblotting protocol was performed. The visualization of all immunoblot detections were performed with horseradish peroxidase-conjugated secondary antibodies (ProteinTech Group) and enhanced chemiluminescence (ECL kit, Beyotime Biotechnology, China).

Flow Cytometry

For FACS (fluorescence-activated cell sorting) analysis, HeLa cells were transiently transfected with HA-TgPP2C and/or treated with DRB (100 µmol/L, 6 h). After trypsin/EDTA harvest and PBS washes, the cells were stained with Alexa Fluor 488 Annexin-V/PI (HH-V13241, Invitrogen) following the protocol of the manufacturer. Annexin V has a high affinity for phospholipid-like phosphatidylserine (PS) and is a widely used marker of cell apoptosis^[22]. Propidium iodide (PI), can only intercalate the double-stranded DNA upon loss of membrane integrity and is a good marker of cell viability. Flow cytometry was performed using a FACS Calibure (BD Biosciences) flow cytometer with laser excitation at 488 nm. Fluorescence emission collection of Alexa Fluor 488 and of PI was through a 530/30 nm and 585/42 nm filters, respectively. The data were analyzed using Flowjo software. Only living cells profiled using forward scatter (FSC) and side scatter (SSC) were counted. A minimum of 10,000 cells within the gated region were analyzed.

Statistics Analysis

Data from the rate of apoptosis are expressed as mean±SEM of three independent experiments.

Statistical analysis was performed using Student’s paired *t*-test and the statistical significance was defined as *P*<0.05.

RESULTS

SSRP1 is a Candidate Partner of TgPP2C Identified by Yeast two Hybrid (Y2H) System

Hybrid plasmids pLEX12-TgPP2C and pGAD-GE-SSRP1 were co-transformed into yeast reporter strain L40. The interaction was analyzed by the X-gal assay. As indicated by the blue color of the yeast patches in Figure 1A, LexBD-TgPP2C fusion protein interacted with GAD-SSRP1 fusion protein (yeast patches group 1). Yeasts co-transformed either with the two empty vectors (yeast patches group 2), or each of the two recombinant vectors plus another empty vector (yeast patches groups 3 and 4) were used as negative controls, while those co-transformed with pGAD-Ras and pLEX12-Raf (yeast patches group 5) were used as positive control.

TgPP2C Interacts with SSRP1 in vitro and in HeLa Cells

We next checked whether the Y2H result could

be confirmed at the protein level by assessing the interaction between SSRP1 and TgPP2C. To this end, we performed His-tag pull-down assays using immobilized recombinant His-TgPP2C. Affinity pull-down assay showed that TgPP2C interacted specifically with SSRP1 from extract of HeLa cells (Figure 1B). For co-immunoprecipitation assay, HeLa cells were transfected with HA-TgPP2C or HA-vector plasmids, respectively. Cells expressing the corresponding HA fusion protein and HA polypeptide (Figure 1C) were lysed and incubated with anti-HA antibody. The immune complexes resulting from the co-immunoprecipitation with anti-HA antibody was examined by Western blot analysis. As shown in Figure 1C, endogenous SSRP1 of HeLa cells was co-precipitated with HA-TgPP2C by anti-HA antibody in HA-TgPP2C-overexpressed cells whereas in cells transfected by HA-vector, no SSRP1 was found in the immune complex.

Mapping of the Binding Domain in SSRP1 for its Interaction with TgPP2C

Next, we tried to identify the binding domain of SSRP1 for its interaction with TgPP2C. Three truncated peptides of SSRP1 have been constructed, corresponding respectively to the N-terminal region

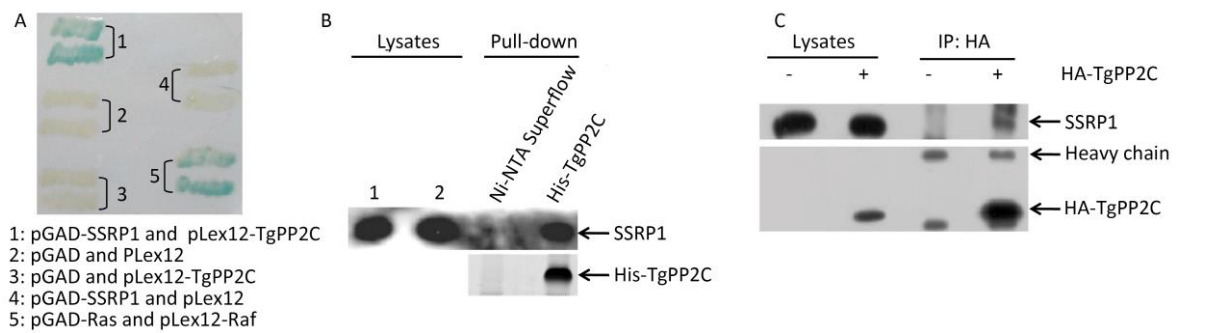


Figure 1. TgPP2C interacted with human SSRP1. (A) TgPP2C-SSRP1 interaction in yeast two hybrid system. Yeasts were transformed by various combinations of plasmids as indicated and analyzed by X-gal assays as described in Materials and methods. Interaction of the two proteins is shown by the blue color of the yeast patches. (B) TgPP2C-SSRP1 interaction in His-tag pull-down assay. HeLa cell lysates were incubated either with Ni-NTA Superflow beads alone (1), or with Ni-NTA beads coated with His-TgPP2C (2) respectively for 4 h at 4 °C. After brief centrifugation, the beads were washed with lysis buffer A, resuspended and boiled in SDS-PAGE sample buffer. Then, the pull-down fractions, together with the lysates (Lysates) were analyzed by Western blotting with antibodies specific to SSRP1 and His. (C) TgPP2C-SSRP1 interaction demonstrated by co-immunoprecipitation assay. HeLa cells transfected with HA-vector or HA-TgPP2C plasmids were harvested for assay. After preclearing by incubation with 20 μ L protein A/G-agarose and 1 μ g mouse IgG at 4 °C for 30 min, HeLa cell lysates were incubated with the mouse monoclonal HA antibody at 4 °C overnight. Total lysates and the immune complexes were analyzed by Western blotting with the specific antibodies. All results are representative of at least three repeated experiments.

(amino acids 1-242, N-SSRP1), the middle region (235-475, M-SSRP1), and the C-terminal region (471-709, C-SSRP1) of the SSRP1 protein (Figure 2A). These truncated peptides of SSRP1 were fused with Gal4 AD in pGAD-GE vector, and analyzed by X-gal assays in two hybrid system for their binding to LexBD-TgPP2C. As shown in Figure 2B, only the C-terminal amino acids 471-709 (pGAD-C-SSRP1) interacted with pLEX12-TgPP2C. Subsequently, the C-terminal region (471-709, C-SSRP1) of SSRP1 was subdivided into three smaller fragments (Figure 2A), and tested similarly using yeast two hybrid system. The X-gal assay showed that the amino acids 471-538 of SSRP1 (C1-SSRP1) was responsible for its interaction with TgPP2C (Figure 2C).

TgPP2C Negatively Regulated Cell Apoptosis

In order to identify the biological function of TgPP2C, especially its role in cell apoptosis, we tested the effects of TgPP2C expression on the apoptosis rate of HeLa cells. The cell death rate was

measured by flow cytometry. The Figure 3A was a representative result of three repeated experiments. The results in Figure 3A and 3B demonstrated that the expression of TgPP2C in HeLa cells significantly reduced the cell death rate, suggesting that the parasitic PP2C might have an inhibitory effect on the apoptosis of cells.

TgPP2C Nnegatively Regulated DRB-induced Cell Apoptosis Probably by Interaction with SSRP1

It has been reported that CKII kinase is necessary for viability and animal embryogenesis^[23-24]. Inhibition of CKII activity with a specific inhibitor DRB^[25-26] induced cell apoptosis^[27-28]. Furthermore, the Serine 510 residue of SSRP1, whose phosphorylation by CKII is important for its DNA-binding activity, is located in the C1-SSRP1 region (amino acids 417-538) responsible for its interaction with TgPP2C^[29]. Therefore, we asked if TgPP2C-SSRP1 interaction may be involved in DRB-induced apoptosis, and if the phosphorylation of

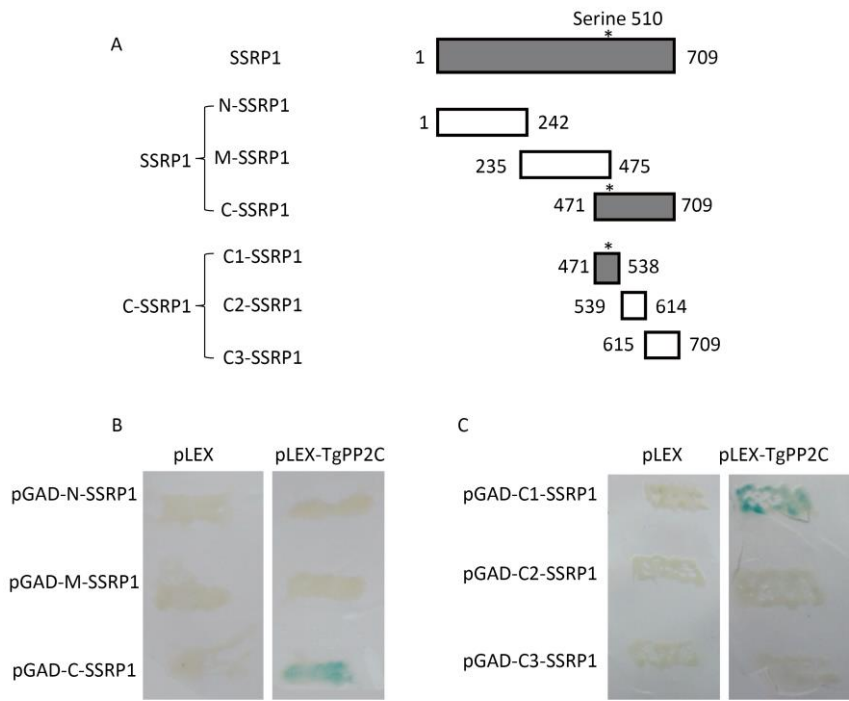


Figure 2. Mapping of TgPP2C binding domain in SSRP1 using yeast two-hybrid system. (A) A schematic presentation of SSRP1 truncated peptide fragments. The grey boxes represent the full-length SSRP1 and SSRP1 truncated peptides capable of binding to TgPP2C, and the white box represent those incapable of interaction with TgPP2C. *: Serine 510 of SSRP1, a target site for CKII phosphorylation. (B) X-gal analysis of interaction between TgPP2C and the N-, M-, and C-SSRP1 fragments. (C) The interactions of TgPP2C with C1-, C2-, and C3-SSRP1 fragments. All results are representative of at least three repeated experiments.

SSRP1 by CKII could impact on PP2C-SSRP1 interaction. To this end, we tested first whether the expression of TgPP2C in HeLa cells may affect the apoptosis induced by DRB treatment. HeLa cells transfected with HA-tagged TgPP2C expression vector or empty vector were treated with or without 100 μ mol/L DRB for 6 h. A fraction of these cells was lysed and subjected to Western blotting analysis to examine the activation level of caspase-3, the marker of cell apoptosis. As shown in Figure 4A, in cells treated with DRB, in comparison with control (DMSO treated) cells, the level of the cleaved caspase-3 was dramatically increased (lane 2 compared to lane 1), indicating a higher level of activation of this enzyme and an apoptotic state of the cells. Interestingly, in cells expressing TgPP2C, the cleavage of caspase-3 by DRB treatment was significantly reduced compared to cells without TgPP2C expression (lane 3 compared to lane 2). This result suggested that the expression of TgPP2C in the cells may have an inhibitory effect on cell apoptosis induced by DRB. In order to confirm this result, another half of the cells treated with DRB were subjected to flow cytometry analysis to assess the death rate of cells. Our results clearly

demonstrated that the expression of TgPP2C in HeLa cells inhibit the cell apoptosis induced by DRB, and that the expression of TgPP2C exerted an inhibitory effect on cell apoptosis (Figure 4B, 4C).

Next, we tested if DRB could affect the binding between TgPP2C and SSRP1. His-tag pull-down assays have been performed using His-PP2C recombinant protein purified with Ni-NTA Superflow nickel columns, and incubated with cell lysates from HeLa cells treated or not previously with 100 μ mol/L DRB. The results in Figure 4D demonstrated that TgPP2C-SSRP1 interaction was significantly increased by DRB treatment, suggesting that the phosphorylation of SSRP1 by CKII might inhibit its interaction with TgPP2C. Giving that the Serine 510 on SSRP1, which is a target site for CKII phosphorylation, is located in the binding domain for TgPP2C interaction, it would be possible that its phosphorylation might bother the interaction with TgPP2C.

DISCUSSION

Despite the considerable progress made by recent studies on the motility of *T. gondii* and its mechanisms of invasion of host cells, many blind spots

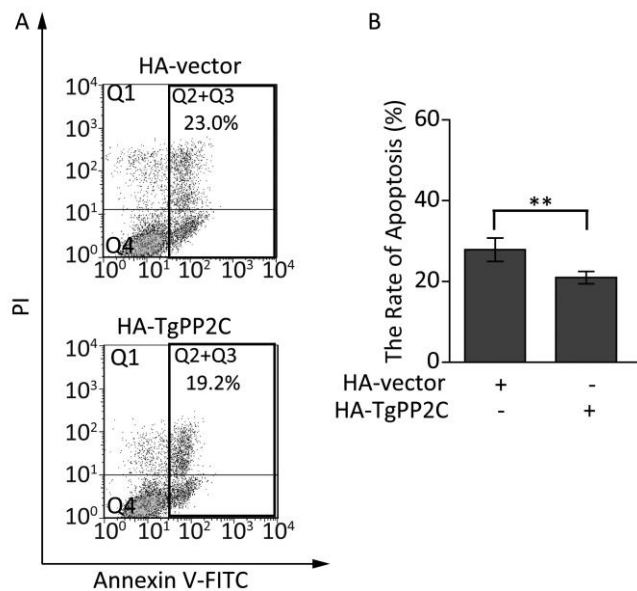


Figure 3. TgPP2C inhibited HeLa cell apoptosis. (A) Flow cytometry analysis of the cell death rate in HeLa cells expressing HA-TgPP2C revealed by Alexa Fluor 488 Annexin-V/PI staining. HeLa cells were transiently transfected with HA-vector or HA-TgPP2C plasmids. After 48 h of the expression of these plasmids, the cells were harvested and stained with Alexa Fluor 488 Annexin-V/PI to analyze the death rate using a FACS Calibure flow cytometer. (B) The average cell death rate analysis of three repeated experiments. The data were shown as mean \pm SEM. **: $P<0.05$.

remain to be enlightened regarding the whole life cycle of the parasite, particularly the strategy and mechanism used by the parasite to control its latency and replication in host cells. More importantly, a recent study, using a new technique to knock out individually each of the parasitic genes considered to be critical for parasite invasion, indicated that the knocking out of none of these genes could affect the invasion ability of the

parasite^[30]. This study raises doubts about current strategies of drug development aiming to block the parasite invasion, and highlight the importance of searching alternative ways to combat the parasite infection.

One common response of host to parasite infection from viruses, bacteria, and eukaryotic pathogens, is apoptosis, which allows to eliminate the infectious agents rapidly by macrophages^[17]. On

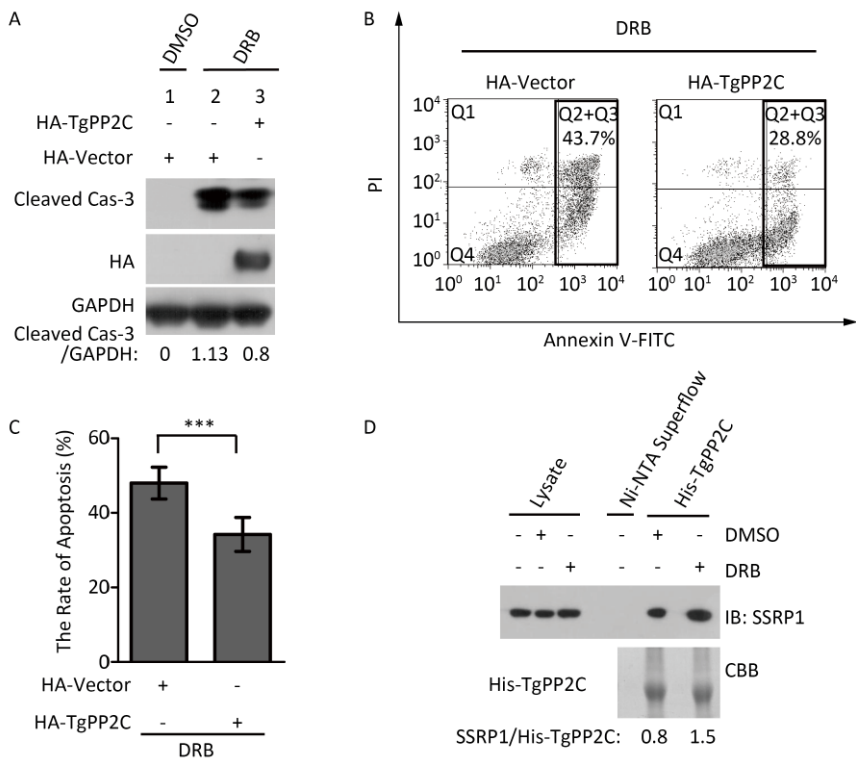


Figure 4. TgPP2C inhibited DRB-induced cell apoptosis and interacted with SSRP1. (A) Western blotting analysis of the levels of cleaved Caspase-3 in HA-TgPP2C-expressed and/or DRB (100 μ mol/L)-treated HeLa cells. Cleaved Cas-3/GAPDH values indicated at the bottom of the figure represent the ratio between the gray values of Cleaved Cas-3 and GAPDH protein bands. (B) Flow cytometry analysis of the cell death rate after DRB treatment in HeLa cells expressing HA-vector or HA-TgPP2C. HeLa cells transiently transfected with HA-vector or HA-TgPP2C plasmids were treated with DRB (100 μ mol/L) for 6 h. After trypsin/EDTA harvest and PBS washes, the cells were stained with Alexa Fluor 488 Annexin-V/PI to analyze the death rate using a FACS Calibure flow cytometer. (C) The average cell death rate analysis of three repeated experiments. The data were shown as mean \pm SEM. ***: $P<0.01$. (D) His-tag pull-down assay analyzed the interaction of TgPP2C with SSRP1 after DRB (100 μ mol/L) treatment. The control untreated HeLa cells or DMSO-, DRB-treated HeLa cells were incubated with Ni-NTA Superflow beads alone or with Ni-NTA beads coated with His-TgPP2C for 4 h at 4 $^{\circ}$ C. After brief centrifugation, the beads were washed, resuspended and boiled in SDS-PAGE sample buffer. Then, the pull-down fractions, together with the lysates (Lysates) were analyzed by Western blotting with SSRP1 antibody. SSRP1/His-TgPP2C values indicated at the bottom of the figure represent the ratio between the gray values of SSRP1 pulled down and recombinant His-TgPP2C used. The recombinant His-TgPP2C was stained with CBB.

the other hand, it has been shown that the microorganisms including *T. gondii* protect host cell from apoptosis induced by a wide range of apoptotic inducers which may provide the possibility of survival for parasite in host cells facilitating its replication or latent infection^[15-16]. *T. gondii* has been suggested to establish an anti-apoptotic condition in a variety of cell types through regulation of several signal pathways with its kinases and a phosphatase^[17]. Previous reports showed that *Toxoplasma* prevented its host cells from apoptosis via promotion of NF- κ B-dependent expression of anti-apoptotic genes^[31-32]. The host cells infected with *T. gondii* exhibit a reduced activation of the caspase cascade after induction of apoptosis, including the lower activation of caspase-8, caspases-9, and caspase-3^[33-35]. Consistent with these results, in our study, the enforced expression of phosphatase TgPP2C of *T. gondii* reduced the amount of cleaved caspase-3 in DRB-treated HeLa cells (Figure 4A), indicating the decreased activation of caspase-3 and the anti-apoptotic role of TgPP2C in HeLa cells under the treatment of apoptotic inducer DRB.

TgPP2C, as the most abundant serine threonine phosphatase of the parasite^[19], plays a very important role in the regulation of parasite growth^[19]. Moreover, PP2C in other organisms have been shown to regulate cell survival and apoptosis^[4]. These findings hint at a major function of this parasitic enzyme in the control of the parasite growth by regulation of the fate of host cells. During the process of parasite invasion, TgPP2C was released and translocated to the host nucleus^[18], suggesting a possible role of this protein linked to the host genome. In this context, the identification of SSRP1 by the present study as a TgPP2C interacting partner turns out to be very meaningful.

SSRP1, by binding to DNA, can alter the structure of chromatin, and regulate the replication and repair of the host genome, as well as the expression of genes^[21]. Furthermore, it is a substrate of CKII, a kinase regulating the cell survival and apoptosis^[29].

Combining yeast Two-hybrid, with *in vitro* pull-down assay, and co-immunoprecipitation, we clearly demonstrated that TgPP2C interacted with SSRP1 (Figures 1 and 2), and that the expression of TgPP2C reduced the DRB-induced cell apoptosis (Figure 4). The inhibitory effect of TgPP2C on DRB-induced cell apoptosis might be mediated by the direct interaction between TgPP2C and SSRP1,

which could offset the inhibitory effect of DRB on the phosphorylation of SSRP1 by CKII. On the other hand, the effect of TgPP2C on cell apoptosis is not specific to DRB treatment, since it could also be observed in cell non-treated with DRB, although in a more moderate extent (Figure 3). Therefore, the action of TgPP2C might not be related specifically to the regulation of the SSRP1 phosphorylation by CKII. Instead, the interaction of TgPP2C with SSRP1 might exert an effect similar to that by CKII phosphorylation on SSRP1, which is to enhance the binding of the latter to damaged DNA and facilitate the DNA repair. Alternatively, TgPP2C-SSRP1 interaction may also modulate the binding of SSRP1 to DNA and regulate the expression of genes involved in cell survival and apoptosis. Figure 5 is a schematic presentation of our hypothesis. Future studies should aim to elucidate the precise mechanism by which TgPP2C-SSRP1 interaction is involved in the inhibition of cell apoptosis.

In conclusion, in the present study, we demonstrate that TgPP2C interacts with mammalian SSRP1 through a C-terminal domain (amino acids 471-538) of the latter. Furthermore, TgPP2C negatively regulates HeLa cells apoptosis possibly by interacting with SSRP1 and enhancing its activity.

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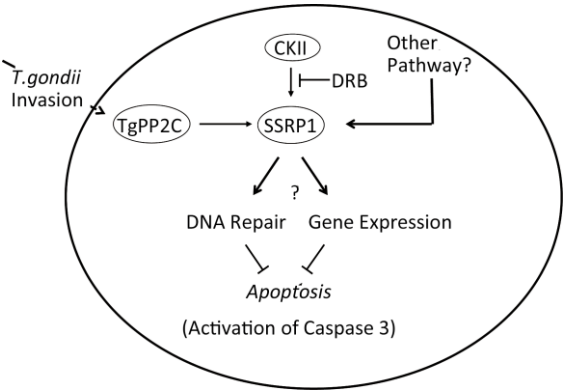


Figure 5. Possible involvement of TgPP2C-SSRP1 interaction in the regulation of cell apoptosis.

AUTHOR CONTRIBUTION

GAO Xue Juan designed this work and drafted the manuscript; FENG Jun Xia carried out most of the experiments; ZHU Sen carried out yeast two-hybrid analysis; LIU Xiao Hui carried out plasmids construction; TARDIEUX Isabelle provided TgPP2C plasmid and revised the manuscript; LIU Lang Xia designed this work and revised the manuscript.

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